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Vaccination To Induce Antibodies Blocking the CX3C-CX3CR1 Interaction of Respiratory Syncytial Virus G Protein Reduces Pulmonary Inflammation and Virus Replication in Mice[▽]

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Respiratory syncytial virus (RSV) infection causes substantial morbidity and some deaths in the young and elderly worldwide. There is no safe and effective vaccine available, although it is possible to reduce the hospitalization rate for high-risk children by anti-RSV antibody prophylaxis. RSV has been shown to modify the immune response to infection, a feature linked in part to RSV G protein CX3C chemokine mimicry. This study determined if vaccination with G protein polypeptides or peptides spanning the central conserved region of the G protein could induce antibodies that blocked G protein CX3C-CX3CR1 interaction and disease pathogenesis mediated by RSV infection. The results show that mice vaccinated with G protein peptides or polypeptides containing the CX3C motif generate antibodies that inhibit G protein CX3C-CX3CR1 binding and chemotaxis, reduce lung virus titers, and prevent body weight loss and pulmonary inflammation. The results suggest that RSV vaccines that induce antibodies that block G protein CX3C-CX3CR1 interaction may offer a new, safe, and efficacious RSV vaccine strategy.

Human respiratory syncytial virus (RSV) is an important and ubiquitous respiratory virus causing serious lower respiratory tract diseases in infants and young children and substantial morbidity and mortality in the elderly and immunocompromised (7, 11, 20, 21). Despite substantial efforts to develop safe and effective RSV vaccines, none have been successful. The first RSV candidate vaccine, a formalin-inactivated alum-precipitated RSV (FI-RSV) preparation, did not confer protection and was associated with a greater risk of serious disease with subsequent natural infection (9, 60). Live attenuated and inactivated whole virus vaccine candidates have also failed to protect, as they were either insufficiently attenuated or demonstrated the potential for enhanced pulmonary disease upon subsequent RSV infection (6, 37, 39, 41, 45). Similarly, subunit vaccine candidates, such as purified F protein and a prokaryotically expressed fusion protein comprising a fragment of the RSV G protein (residues 130 to 230) fused by its N terminus to the albumin binding domain of streptococcal protein G (designated BBG2Na), have been shown to be inadequate (8, 33, 37, 41). The specific reasons for RSV vaccine failure remain to be answered but could be related to RSV-mediated circumvention of immunity and, more broadly, to the lack of durable immunity elicited in response to natural RSV infection, as people of all ages may experience repeated infections and disease throughout life (3, 41, 45).

Evidence indicates that the RSV F protein is important in inducing protective immunity (19, 38), but studies evaluating a BBG2Na vaccine candidate in combination with different adjuvants and by different routes of administration have shown a role for G protein in protection against RSV in rodents (4, 10, 17, 32, 43, 44, 49, 51). The structural elements of the G protein fragment in the BBG2Na vaccine candidate implicated in protective efficacy were mapped, and five different B-cell epitopes were determined, i.e., residues 145 to 159, 164 to 176, 171 to 187, 172 to 187, and 190 to 204 (44, 48). Interestingly, immunogenicity of peptides with residues 145 to 159 was dependent on the orientation of the covalent peptide coupling to the carrier proteins, as mice vaccinated with C-terminally coupled peptides developed protective antibody titers, whereas mice vaccinated with N-terminal peptides did not. The focus of the BBG2Na vaccine studies centered on development of protective neutralizing antibodies, and the studies showed that vaccination or priming with the G protein fragment in BBG2Na did not induce signs of enhanced pulmonary pathology (17, 42, 46, 50).

Despite the strong evidence that G protein peptides and polypeptides can induce protective immunity, the G protein has also been implicated in disease pathogenesis (30, 40, 41, 54). One of the disease mechanisms linked to the G protein is CX3C chemokine mimicry (56). RSV G protein has marked similarities to fractalkine, the only known CX3C chemokine, including similarities in structural features (56). Both G protein and fractalkine exist as membrane-bound and secreted forms, and both contain a CX3C chemokine motif that can bind to the fractalkine receptor, CX3CR1 (15, 27). Fractalkine functions to recruit immune cells to sites of inflammation, in particular, $CX3CR1^+$ leukocytes, which include subsets of NK cells and $CD4^+$ and $CD8^+$ T cells (23). RSV G protein has

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TABLE 1. RSV G protein peptides designed to immunize mice for antisera

Peptide	Partial G protein aa sequence[a]
	171 182 186 201
LH WT	VPCSICSNNPTCWAICKRIPNKKPGKKTTTKP
LH RT32	VPCSICSNNPTC
LH RT33	TCWAICKRIPNK
LH RT34	NKKPGKKTTTKP
LH RT33+ala	TCAAACKRIPNK
LH D-1	TCWA CKRIPNKK
LH D+1	TCWAIACKRIPNK
LH 93	INGKWIILLSKF
LH G174-187	SICSNNPTCWAICK

[a] The location of the CX3C motif (CWAIC) in the G protein is underlined and compared to G protein peptides outside the CX3C motif (LH RT32, LH RT34, and LHL393), peptides containing the CX3C motif (LH WY and LH RT33), and peptides with a single aa deletion (LH D-1) or insertion (LH D+1).

been shown to have fractalkine-like leukocyte chemotactic activity *in vitro* (56). *In vivo*, RSV G protein acts as a fractalkine antagonist, modulating the immune response to infection by inhibiting fractalkine-mediated responses by altering the trafficking of $CX3CR1^+$ cells and modifying the magnitude and cadence of cytokine and chemokine expression (23, 55). Infection of mice with a mutant RSV lacking the CX3C motif leads to a substantial increase of pulmonary NK cells and $CD4^+$ and $CD8^+$ cells compared to infection with wild-type RSV (23). This suggests that G protein CX3C-CX3CR1 interaction contributes to immune evasion and may contribute to disease pathogenesis. Thus, G protein CX3C interaction with CX3CR1 is an important target for disease intervention strategies against RSV infection.

In the present study, we investigated a new RSV vaccine strategy, using G protein polypeptide and peptide vaccination to generate antibodies reactive to the central conserved cysteine noose region of the G protein to block G protein CX3C motif interaction with CX3CR1. We hypothesize that vaccines inducing G protein-CX3CR1 blocking antibodies will prevent much of the RSV G protein-mediated immune modulation and disease pathogenesis. Our results show that antibodies induced by the central conserved noose region of the G protein block G protein binding to CX3CR1, prevent body weight loss indicative of disease pathogenesis, decrease pulmonary inflammation, and decrease lung virus titers compared to antibodies reactive to N- and C-terminal regions of the G protein. These results suggest that a vaccine strategy to induce G protein CX3C-CX3CR1 blocking antibodies may be useful to prevent G protein-mediated immune modulation and disease pathogenesis.

MATERIALS AND METHODS

Peptide and polypeptides. G protein polypeptides or peptides spanning different regions of the G protein were designed for vaccination (Table 1; see Fig. 2). The G peptides were commercially synthesized by liquid-phase peptide synthesis (GenScript, Piscataway, NJ) and dissolved in dimethyl sulfoxide (DMSO) (Sigma-Aldrich, St. Louis, MO) to a concentration of 2.5 mg/ml. The G polypeptides were expressed and purified from *Escherichia coli*. The G gene fragments were synthesized, codon optimized (IDT, Coralville, IA) for expression in bacteria, cloned into the expression vector pRsetA (Invitrogen, Carlsbad, CA), and expressed in *Escherichia coli*. The proteins were purified by immobilized metal affinity chromatography as previously described (31).

Transfection and selection of human 293 cells expressing CX3CR1. Human 293 cells (CRL-1573; ATCC) were transfected with pcDNA3.1 expression plasmids (Invitrogen Corp., Carlsbad, CA) encoding CX3CR1. Plasmid inserts were derived from genomic DNA by high-fidelity PCR amplification (Invitrogen) and were sequenced bidirectionally. After G418 selection for at least 3 weeks, stable receptor expression was verified by flow cytometry. In brief, stably transfected cells (CX3CR1.293 cells) were stained with a fluorescein isothiocyanate (FITC)-conjugated anti-CX3CR1 monoclonal antibody (MAb 2A9) obtained from MBL International (Nagoya, Japan). Cell sorting was performed using a DakoCytomation MoFLo high-speed cell sorter after gating of dead cells by use of propidium iodide and correction of results for nonspecific staining by use of isotype antibody controls.

Native RSV G protein purification. Native G protein was purified from RSV A2-infected Vero cells by affinity chromatography as previously described (56). Briefly, RSV-infected (multiplicity of infection [MOI] = 1) Vero E6 cells (CRL-1586; ATCC) were collected at day 5 postinfection, and the cell pellets were resuspended in cold phosphate-buffered saline (PBS) containing Complete protease inhibitor (Roche, Indianapolis, IN). Triton X-100 (Sigma) was added to the cell pellet to a final concentration of 1.0%, and the cell slurry was stirred at room temperature for 30 min. Lysate was subjected to sonication at 85% power for 6 cycles of 60 s, with resting for 5 min between cycles, followed by centrifugation at 10,000 × *g* for 15 min. The supernatant was collected, filtered through a 0.2-μm filter, and applied to a 5-ml Hi-Trap *N*-hydroxysuccinimide (NHS)-activated column (GE Healthcare, Amersham Biosciences) coupled to an anti-RSV G monoclonal antibody (clone 131-2G) according to the manufacturers' instructions. The column was equilibrated with 2 column volumes of PBS plus 0.2% *N*-octyl-β-glycoside by fast-performance liquid chromatography (FPLC) (GE Healthcare, Amersham Biosciences) at a flow rate of 2 ml/minute. Lysate was loaded at a flow rate of 1.0 ml/minute, and the column was washed with 4 column volumes of PBS plus 0.2% *N*-octyl-β-glycoside and eluted with 4 column volumes of 0.1 M glycine, 1% *N*-octyl-β-glycoside, pH 2.7, with collection of 10 2-ml fractions. The fractions were neutralized with 0.3 ml of 2 M Tris, pH 8.0, and those fractions containing the G protein (determined by UV absorption during FPLC and by Western blot analysis) were pooled and dialyzed overnight at 4°C against PBS, pH 7.4. This purification process yields highly purified G protein with no detectable F protein by Western blot analysis and no detergent after dialysis.

G polypeptide/peptide vaccination. Four- to 6-week-old specific-pathogen-free female BALB/c mice were purchased from Charles River Laboratories, housed in microisolator cages, and fed sterilized water and food ad libitum. The studies were reviewed and approved by the university institutional animal care and use committee. All peptides were conjugated to maleimide-activated keyhole limpet hemocyanin (KLH; ThermoScientific, Rockford, IL) for vaccination according to the manufacturer's protocol. KLH-conjugated peptides were emulsified 1:1 with Montanide ISA 720 (Seppic, Paris, France), and mice were immunized intramuscularly (i.m.) with a total of 50 μg vaccine/mouse in the hind quarters. G protein polypeptides were emulsified 1:1 with TiterMax (Sigma), and mice were immunized i.m. with a total of 50 μg vaccine/mouse in the hind quarters. Vaccinated mice were boosted at 14 days postvaccination. A single boost was needed for polypeptide vaccination, and two boosts were required for peptide vaccination to achieve a G protein-reactive antibody titer of ≥3 standard deviations (SD) above the background determined by enzyme-linked immunosorbent assay (ELISA). The antisera from control and G protein peptide/polypeptide-vaccinated mice were collected and stored at −80°C for blocking antibody assays.

ELISA. The antibody titers in sera from vaccinated mice were determined using a modified indirect ELISA as previously described (24). Briefly, flat-bottom microtiter plates (Corning, Corning, NY) were coated with native G protein or immunizing antigen overnight at 4°C. Serial dilutions of sera in PBS were added to the wells and incubated for 1 h at 37°C. The plates were washed three times with washing buffer (PBS containing 0.05% Tween) and incubated for 1 h at 37°C with alkaline phosphatase-conjugated goat anti-mouse IgG (H+L) (Millipore, Temecula, CA). After being washed, the plates were developed with pNpp substrate (Pierce) as indicated by the manufacturer.

CX3C-CX3CR1 binding and inhibition assays. Immunoglobulin G (IgG) was purified from the sera of vaccinated mice by use of an immobilized protein G column (Pierce), as indicated by the manufacturer, to allow for normalizing for the same concentrations of IgG and to remove endogenous CX3CL1 and other serum factors which might compete with G protein for binding to CX3CR1. In the blocking studies, dilutions of purified IgG antibodies were made in PBS (0.01, 0.1, 1, 2, and 5 μg/ml) and were preincubated with 500 nM of purified RSV G protein for 1 h at 4°C. A monoclonal antibody isotype control and monoclonal antibody from clone 131-2G (reactive to RSV A2 G protein) were used as negative and positive controls, respectively, at the same IgG concentrations.

CX3CR1.293 or untransfected 293 cells were washed in PBS and plated in a round-bottom 96-well plate at 2×10^5 cells/well. The cells were washed again, resuspended in PBS containing anti-human CD32 (Fc block; Millipore) at 1 μg/ml, and incubated at 4°C for 15 to 20 min. The cells were resuspended in the premix of purified IgG and RSV G protein, 2.5 μg/ml heparin (Sigma) was added to prevent nonspecific binding, and the cells were incubated for 45 min at 4°C. Cells were subsequently washed in PBS containing 0.2% bovine serum albumin (BSA; Sigma) (fluorescence-activated cell sorting [FACS] buffer) and incubated with Alexa 488 (Molecular Probes, Eugene, OR)-conjugated anti-G-protein monoclonal antibody (clone 130-2G) for 30 min at 4°C. The optimal concentration of IgG for G protein CX3C-CX3CR1 inhibition ranged from 1 to 2 μg/ml. The percentage of cells binding to G protein was determined by FACS analysis, using a BD LSRII flow cytometer. The percent specific binding of G protein to CX3CR1 was calculated by the formula (percent G protein binding to CX3CR1.293 cells) − (percent G protein binding to 293 cells) × 100, as previously described (56). The percent specific inhibition of G protein binding to CX3CR1 was calculated by the formula [(percent G protein binding to CX3CR1.293 cells in the presence of blocking antibody) − (percent G protein binding to 293 cells in the presence of blocking antibody)]/[(percent G protein binding to CX3CR1.293 cells in the presence of nonspecific antibody) − (percent G protein binding to 293 cells in the presence of nonspecific antibody)] × 100.

Serum antibody inhibition of G protein-mediated leukocyte chemotaxis. The ability of serum antibodies to inhibit RSV G protein-mediated leukocyte migration was determined by chemotaxis assay, using a modified Boyden chamber as previously described (24). To remove endogenous CX3CL1 and other serum factors that may affect leukocyte migration, serum antibodies were isolated by use of protein A/G. Briefly, serum was diluted 1:20 in Dulbecco's PBS (D-PBS; Invitrogen), and an equal mixture of protein A- and protein G-coated magnetic beads (Dynal; Invitrogen, Carlsbad, CA) was added to each sample. Samples were incubated at 4°C for 1 h, and the serum was decanted to a new tube for repeated Ig isolation using new protein A/G-coated beads. Ig-bound protein A/G magnetic beads were washed twice with D-PBS, and the Ig was eluted with 0.1 M glycine, pH 2.2. The procedure was repeated twice. After three elutions, the eluates were pooled and dialyzed against D-PBS overnight at 4°C. Dialyzed serum antibodies were standardized by protein concentration, diluted in serum-free Dulbecco's modified Eagle's medium (DMEM; Gibco), and added to the lower well of a chemotaxis chamber (Neuro Probe, Gaithersburg, MD) at a concentration of 2.25 μg/ml. Purified RSV G protein (56) diluted in serum-free DMEM was added to the lower well at a concentration of 22.5 μg/ml. Peripheral blood leukocytes were purified from three healthy adult volunteers by Ficoll gradient separation (MP Biomedicals, Solon, OH) and then were washed and resuspended in serum-free DMEM, and 1.8×10^5 freshly isolated lymphocytes were added to the upper wells, which were separated from the lower wells with an 8-μm-pore-size filter. The chamber was incubated for 12 h at 37°C, and the number of viable cells that migrated into the lower chamber was determined. Negative and positive control wells contained serum-free DMEM and anti-G monoclonal antibody (clone 131-2G), respectively. The chemotactic index (CI) was defined as (the number of viable cells migrating toward RSV G protein)/(the number of viable cells migrating toward serum-free DMEM), and the percent inhibition of chemotaxis was defined as [1 − (CI toward G protein and serum/CI toward G protein)] × 100). Each serum sample was analyzed in duplicate over three different assays, using three different lymphocyte donors for each assay.

Serum antibody RSV microneutralization assay. Sera obtained from G protein peptide- and polypeptide-vaccinated mice were evaluated using an established plaque reduction assay (61). Briefly, G protein peptide/polypeptide serum, RSV/A2-hyperimmune serum, and sera from naïve mice were heat inactivated at 56°C for 30 min, and serial twofold dilutions, starting at a dilution of 1:100, were made in MEM containing 2% heat-inactivated fetal bovine serum (FBS). All sera were tested in the presence of 10% guinea pig complement (BioWittaker Cambrex Co., Walkersville, MD). Equal volumes of serum dilutions and RSV strain A2 previously titrated to yield 100 PFU/50 μl/well of final mixture were incubated at 37°C and 5% CO_2 for 1 h. Confluent monolayers of Vero cells prepared in 96-well plates were infected with 50 μl/well, in triplicate, of the serum-virus mixture. After virus adsorption for 2 h at 37°C, the cell monolayers were overlaid with 0.1% methylcellulose in MEM with 2% FBS at 100 μl/well. Plates were incubated at 37°C and 5% CO_2 for 3 days. The cells were then fixed with a 50%-50% methanol-ethanol mixture and incubated with a mouse monoclonal antibody specific for RSV F protein (clone 131-2A) followed by a secondary goat anti-mouse IgG antibody conjugated with peroxidase (KPL, Gaithersburg, MD). Plaques were developed using 100 μl/well of 3,3′-diaminobenzidine (0.5 mg/ml; 0.01% H_2O_2) at room temperature for 5 to 10 min. Plaques were counted using a dissecting microscope. Titers were calculated from the averages of triplicate sample wells and expressed as the percent plaque reduction relative to that of the naïve serum control.

Disease pathogenesis. The G protein peptides (LH D-1, LH RT32, and LH WT) and polypeptides (G1, G2, and G3) used for the disease pathogenesis study were selected based on their ability to induce antibodies that more effectively blocked G protein CX3C-CX3CR1 interaction. Vaccination of the mice ($n \geq 5$/group) was performed as described above (G polypeptide/peptide vaccination). All control groups ($n \geq 5$) were vaccinated i.m. with 10^6 PFU RSV A2 or uninfected Vero E6 cell lysate equivalent. For the study, mice were challenged intranasally (i.n.) with 10^6 PFU RSV A2 after the last vaccination. The body weight of mice was determined every day after virus challenge, i.e., days 0 to 6. Lung histopathological examination was performed for each group of vaccinated mice, where lung tissues were fixed in 10% buffered formalin, embedded in paraffin, sectioned, and stained with hematoxylin and eosin prior to light microscopy observation, using previously described methods (1). Multiple sections from each tissue block were analyzed by light microscopy as described previously (1). Peribronchiolitis was defined as inflammatory cells, mostly lymphocytes and macrophages, that accumulate around the periphery of small airways. Alveolitis was defined as inflammatory cells, mostly macrophages and neutrophils, within the air space. Interstitial pneumonitis was defined as thickening of the alveolar wall, associated with an influx of inflammatory cells of various types. Each parameter was scored separately under blinded conditions and was assigned a score based on a scale of 0 (no inflammation) to 4 (maximum inflammation).

Lung virus titers. RSV lung virus titers in vaccinated and control mice were determined as previously described (57). Briefly, lungs were aseptically removed from mice at days 2, 4, and 6 post-RSV challenge (10^5 PFU/mouse), and individual lung specimens were homogenized at 4°C in 1 ml of serum-free DMEM (Invitrogen) by use of a tissue lyser (Qiagen, Valencia, CA). The supernatants were transferred to a new tube, snap-frozen by liquid nitrogen, and stored at −80°C until they were assayed. For the plaque assay, 10-fold serial dilutions of the lung homogenates were added to 90% confluent Vero E6 cell monolayers. Following adsorption for 1 h at 37°C, cell monolayers were overlaid with DMEM containing 2% FBS (HyClone; Thermo Scientific) and 2% carboxymethylcellulose (Sigma) and incubated at 37°C for 5 days. The plaques were enumerated by immunostaining with monoclonal antibodies against RSV F protein (clone 131-2A).

Statistics. Student's t test for unpaired samples was used to compare the responses between G polypeptide/peptide-immunized groups and control groups of mice. P values of <0.05 were considered significant.

RESULTS

G protein binds to CX3CR1 highly expressed from a stably transfected human 293 cell line. Previously, we showed that the RSV G protein CX3C motif interacts with the CX3CL1-specific receptor CX3CR1, competes with fractalkine (CX3CL1) for binding to CX3CR1, facilitates infection, and alters CX3CL1-mediated responses (23, 56). In this study, we rederived human 293 cells to more highly express CX3CR1 (CX3CR1.293 cells) to allow for improved evaluation of blocking antibodies induced by G protein polypeptide or peptide vaccination. The expression level of CX3CR1 was determined by flow cytometry and showed that >95% of CX3CR1.293 cells expressed CX3CR1, in contrast to untransfected 293 cells (Fig. 1A). Using the rederived CX3CR1.293 cells and comparing binding to that of untransfected 293 cell controls, we showed that native RSV G protein binds to CX3CR1 approximately 10-fold less than CX3CL1 does, and both G protein and CX3CL1 bind to CX3CR1 in a dose-dependent fashion (Fig. 1B). The higher CX3CR1 binding by CX3CL1 than by purified G protein is not surprising, as CX3CL1 is the natural ligand for this receptor. The results show that G protein, like CX3CL1, binds CX3CR1.

Antibody responses generated by G polypeptide/peptide vaccination. Having established an RSV G protein CX3C-CX3CR1 binding model, we determined whether antibodies generated to epitopes spanning the central conserved region of G protein blocked G protein interaction with CX3CR1. Three

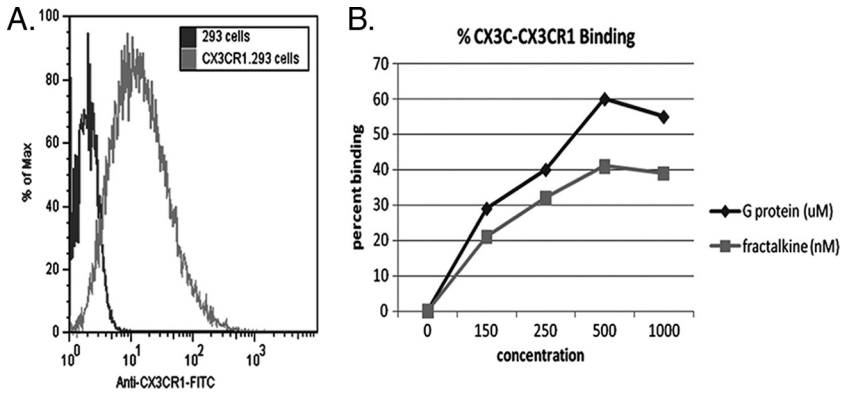

FIG. 1. Expression of CX3CR1 on human 293 cells and fractalkine and G protein binding to CX3CR1. (A) Human 293 cells were stably transfected with a plasmid expressing CX3CR1, and the expression of CX3CR1 was determined by flow cytometry analysis of cells stained by anti-CX3CR1–FITC. (B) Fractalkine (nM) and RSV G protein (μM) binding to CX3CR1-transfected cells occurs in a dose-dependent manner. Data are presented as the percent serum IgG binding to CX3CR1-transfected cells.

G protein polypeptides and nine G protein peptides were carefully designed based on previous findings showing that some G protein peptides spanning the central conserved noose region of the G protein could interfere with G protein binding to CX3CR1 (56). The three G protein polypeptides that were used for immunization corresponded to (i) the internal variable glycosylated region (RSV G1; amino acids [aa] 67 to 147), (ii) the central conserved region (RSV G2; aa 148 to 198), and (iii) the carboxy-terminal variable region (RSV G3; aa 199 to 298) of the G protein of RSV strain A2. A linear representation of the RSV G protein and the localization of G polypeptides are shown in Fig. 2. The following G protein peptides, as shown in Table 1, were designed for vaccination: (i) a wild-type G protein peptide encompassing the G protein CX3C motif (LH WT; aa 171 to 201), (ii) a 12-mer G protein peptide N-terminal to the CX3C motif (LH RT32; aa 171 to 183), (iii) a 12-mer G protein peptide encompassing the G protein CX3C motif (LH RT33; aa 181 to 193), (iv) a 12-mer G protein peptide C-terminal to the CX3C motif (LH RT34; aa 190 to 201), (v) a 12-mer G protein peptide variant of RT33 containing an Ala substitution for Ile in the CX3C motif (LH RT+ALA; aa 181 to 193), (vi) a 12-mer G protein peptide with a deletion of Ile in the CX3C motif (LH D-1; aa 181 to 192), (vii) a 12-mer G protein peptide with an Ala addition in the CX3C motif (LH D + 1; aa 181 to 193), (viii) a heterologous I-Ed-restricted 12-mer peptide from the L protein (amino acids 393 to 405) of RSV, and (ix) a G peptide that has previously been shown to induce a protective immune response (aa 174 to 187) (59).

Using these G protein polypeptides and peptides, BALB/c mice were vaccinated i.m. with individual polypeptide or peptide antigens plus adjuvant. The mice receiving G protein polypeptides were boosted once, and mice receiving G protein

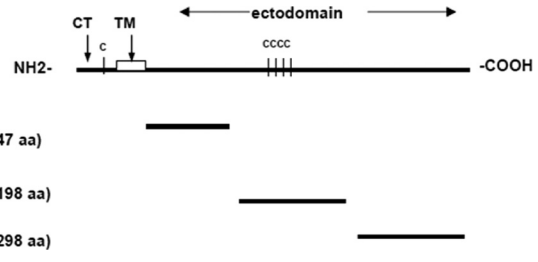

FIG. 2. Linear representation of RSV G protein and G polypeptide fragments. The cysteine noose region in the G protein is indicated with small c's, while the transmembrane and cytoplasmic domains are indicated by TM and CT, respectively.

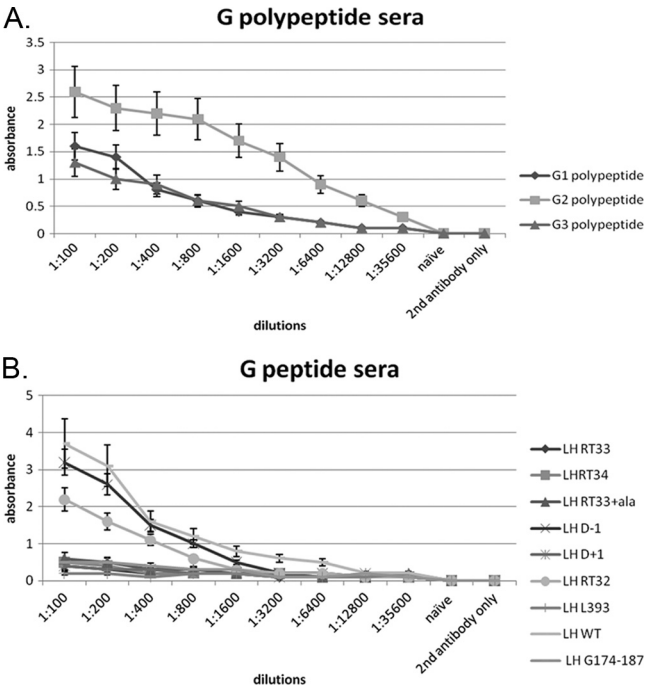

FIG. 3. Titers of sera from mice vaccinated with G polypeptides and G peptides. (A) Mice were immunized with either of three G polypeptides (G1, G2, or G3) adjuvanted with TiterMax at 50 μg antigen per mouse. At 2 to 3 weeks postvaccination, the mice were boosted with the same vaccine antigens. Blood was collected, and antibody titers in the sera were measured by ELISA against the immunizing antigen. (B) Mice were immunized with nine different G peptides (indicated in Table 1) adjuvanted with Montanide at 50 μg antigen per mouse. The mice were boosted twice, at 2-week intervals, with the same antigens. Blood was collected, and serum antibody titers were measured by ELISA against the immunizing antigen.

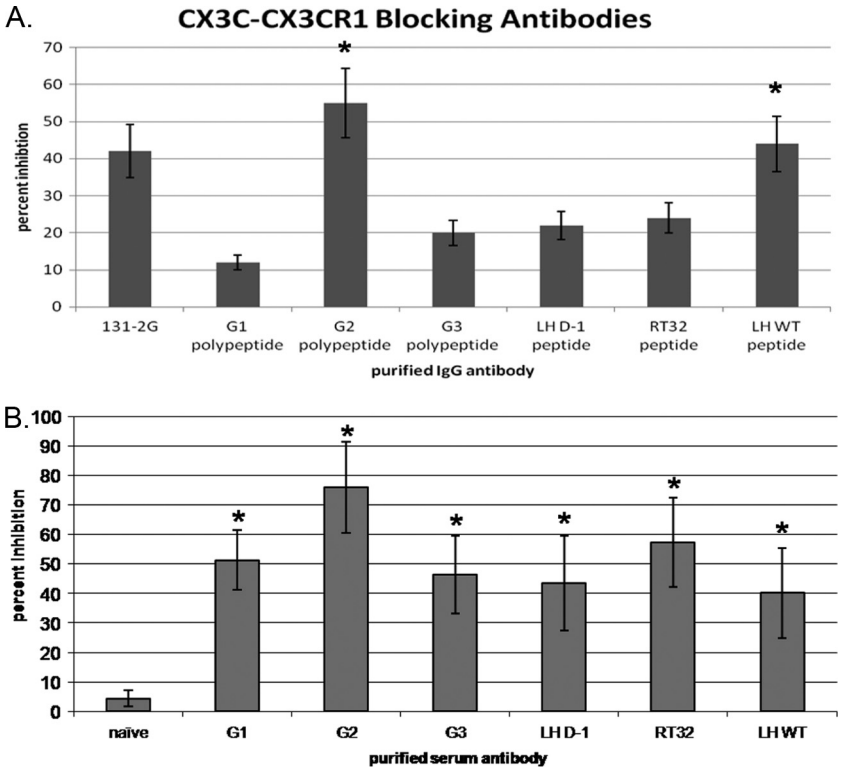

FIG. 4. G protein CX3C-CX3CR1 blocking antibody studies. IgGs purified from antisera derived from mice vaccinated with G protein polypeptides or peptides were evaluated for the ability to prevent native RSV G protein binding to CX3CR1 (A) and to block G protein-mediated leukocyte migration (B). Data are presented as the percent inhibition of RSV G protein binding to CX3CR1.293 cells (A) and the percent inhibition of RSV G protein-mediated leukocyte chemotaxis (B). *, $P < 0.01$ compared to other polypeptides or peptides (A) or compared to serum antibody derived from naïve mice (B).

peptide vaccinations were boosted twice. The serum antibody titers following vaccination or boosting were evaluated by an indirect ELISA 7 days after the final vaccination or boost. All three G protein polypeptide vaccines generated satisfactory antibody responses, with G2 polypeptide-vaccinated mice producing the highest antibody response against native G protein (Fig. 3A). The majority of the G protein peptides failed to induce a substantial antibody response, despite conjugation to KLH, boosting, and the use of adjuvant (Fig. 3B). However, three peptides encompassing or immediately proximal to the G protein cysteine noose region (LHWT, LH RT32, and LH D-1) produced relatively potent antibody responses (Fig. 3B), suggesting that these regions were antigenic. Thus, the three G protein polypeptides (G1, G2, and G3) and three G protein peptides (LH WT, LH RT32, and LH D-1) were chosen for further investigation.

Antibody inhibition of G protein binding to CX3CR1. Serum antibodies generated from the three G protein polypeptides (G1, G2, and G3) and the three peptides (LH WT, LH RT32, and LH D-1) that had the highest antibody titers to native G protein (Fig. 3) were purified by use of immobilized protein G to normalize IgG levels for the G protein CX3C-CX3CR1 blocking studies and to remove endogenous CX3CL1 and other serum factors which might compete with G protein for binding to CX3CR1. Normalized IgG antibodies from the sera of vaccinated mice and MAb clone 131-2G (positive control) all showed various levels of G protein CX3C-CX3CR1 blocking activity (Fig. 4A). However, IgG from G2 polypeptide- and LH WT peptide-vaccinated mice showed the most inhibition, whereas IgG from G1 or G3 polypeptide-vaccinated mice showed minimal (range, 10 to 24%) blocking activity (Fig. 4A). These results show that antibodies reactive to N- or C-terminal sites proximal to aa 148 to 198 in the G protein have minimal G protein CX3C-CX3CR1 blocking activity. Interestingly, IgG from LH WT peptide-vaccinated mice showed similar blocking activity (range, 38 to 52%) to that of IgG derived from sera from G2 polypeptide-vaccinated mice (range, 45 to 65%), indicating that aa 171 to 198 in the cysteine noose region of the G protein are immunogenic and can induce antibodies blocking G protein binding to CX3CR1.

IgG antibodies from mice vaccinated with polypeptides G1, G2, and G3 or with LH WT, LH RT32, or LH D-1 were also examined for the ability to inhibit RSV G protein-mediated leukocyte chemotaxis (Fig. 4B). In all cases, sera from vaccinated mice significantly inhibited chemotaxis toward RSV A2 G protein ($P < 0.01$) compared to antibody from naïve mice, with the greatest inhibition mediated by sera from mice vaccinated with G2 (range, 60 to 92%). In comparison, MAb clone 131-2G generally inhibits RSV G protein-induced chemotaxis by ~50% (data not shown). Given these results and the role of G protein CX3C-CX3CR1 interaction in aspects of disease pathogenesis (23–26, 34, 55), the results suggest that antibodies elicited to this region by vaccination might prevent disease pathogenesis associated with RSV infection.

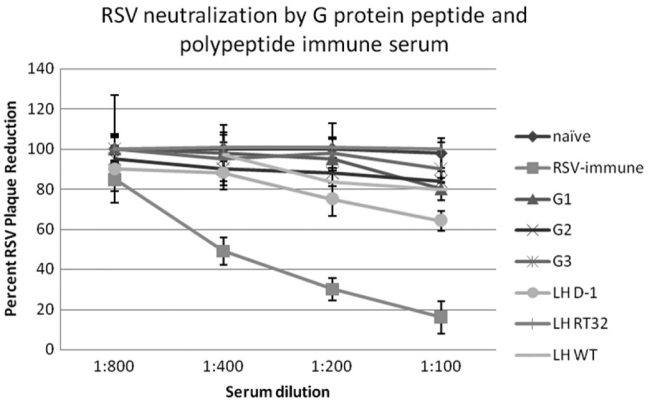

FIG. 5. RSV neutralizing antibody titers in G protein peptide- and polypeptide-vaccinated mice. Sera from G protein peptide (LH D-1, LH RT32, and LH WT)- and polypeptide (G1, G2, and G3)-vaccinated mice were evaluated for neutralizing antibody titers by plaque reduction assay. RSV-immune and naïve sera were used as positive and negative controls, respectively. Data are presented as the percent RSV plaque reduction, calculated from the average for triplicate sample wells and expressed as the percent plaque reduction relative to the naïve serum control.

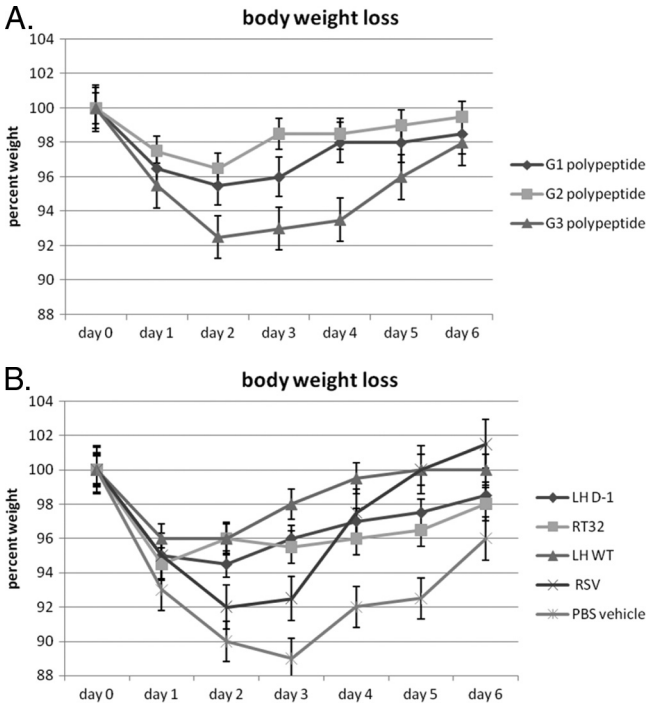

FIG. 6. Body weight loss in G protein polypeptide-, G protein peptide-, and RSV-vaccinated mice or unvaccinated mice challenged with RSV A2. Mice were vaccinated with G protein polypeptides (G1, G2, and G3) (A) or KLH-conjugated peptides (LH D-1, LH RT32, and LH WT) (B) or with RSV A2. Vaccinated and unvaccinated mice were challenged intranasally with 10^6 PFU RSV A2. Data are presented as the percent weight loss and are representative of two independent studies.

Prevention of disease pathogenesis by G polypeptide/peptide vaccination. To determine the association between antibodies that block G protein binding to CX3CR1 and RSV disease severity, mice were vaccinated with G protein polypeptides (G1, G2, and G3) and peptides (LH D-1, LH RT32, and LH WT) that produced effective blocking antibody responses (Fig. 4). Vaccination was performed exactly as in the previous studies, sera were collected from the vaccinated animals 7 days after the final vaccination or booster, and the level of neutralizing antibody, reactivity to native G protein by ELISA, or ability to block G protein CX3C-CX3R1 binding by flow cytometry was determined, using CX3CR1.293 and 293 cells. The serum antibody titers were comparable to those in the previous experiments (Fig. 3; data not shown), and the sera from G2 polypeptide- and LH WT peptide-vaccinated mice blocked G protein CX3C-CX3R1 binding at comparable levels to those in previous studies (Fig. 4; data not shown). No substantial neutralizing antibody titers were detected in the sera of G protein peptide- or polypeptide-vaccinated mice compared to RSV A2 hyperimmune sera (Fig. 5).

Immediately following serum collection at day 7, mice were challenged i.n. with 10^6 PFU RSV A2. The percent body weight loss was determined as a parameter of disease pathogenesis. Body weights were measured daily from day 0 to day 6 postchallenge, and lungs were collected at each time point to determine viral titers and histopathology by hematoxylin and eosin staining. Mice vaccinated i.m. with G protein peptides or polypeptides displayed less body weight loss following RSV challenge than did mice vaccinated i.m. with live RSV or with PBS control (Fig. 6). Among the groups of vaccinated mice, the G2 polypeptide (Fig. 6A)- and LH WT peptide (Fig. 6B)-vaccinated mice showed the least body weight loss. For G2 polypeptide-vaccinated mice, the percent body weight loss was statistically lower ($P < 0.05$) than that for G1 or G3 polypeptide-vaccinated mice at days 2 to 4 postchallenge. For LH WT peptide-vaccinated mice, the percent body weight loss was statistically lower ($P < 0.05$) than that for RT32-, RSV-, or PBS-vaccinated mice at days 2 to 6 postchallenge.

To determine the degree of lung histopathology in the vaccinated mice, the levels of peribronchiolar, perivascular, and interstitial lymphocytic infiltrates, typically associated with RSV-mediated pathogenesis (18), were determined as indicated in Materials and Methods. No lung histopathology was evident at day 0 prior to RSV infection for any vaccination group, indicating that vaccination with G protein peptides or polypeptides did not cause detectable lung pathogenesis (Fig. 7). Considerable histopathology (score range, 3 to 4) was evident in mice treated with PBS vehicle only (Fig. 7A). No substantial lung histopathology (score range, 0 to 1.5) was observed in G2 polypeptide-vaccinated mice (Fig. 7A), LH RT32 peptide-vaccinated mice (score range, 0.5 to 1.5) (Fig. 7B), or LH WT peptide-vaccinated mice (score range, 0.5 to 1.5) (Fig. 7B) at days 4 and 6 postchallenge compared to the other vaccination groups (score range, 2.5 to 3.5). These results are consistent with the limited body weight loss observed for these vaccinated mice compared to the other vaccinated groups (Fig. 6). These results are also consistent with earlier findings which showed that G protein CX3C-CX3CR1 interaction is important for RSV vaccine-enhanced disease pathogenesis (26) and suggest that antibodies which block G protein CX3C-CX3CR1 interaction effectively reduce or inhibit parameters of disease pathogenesis in mice.

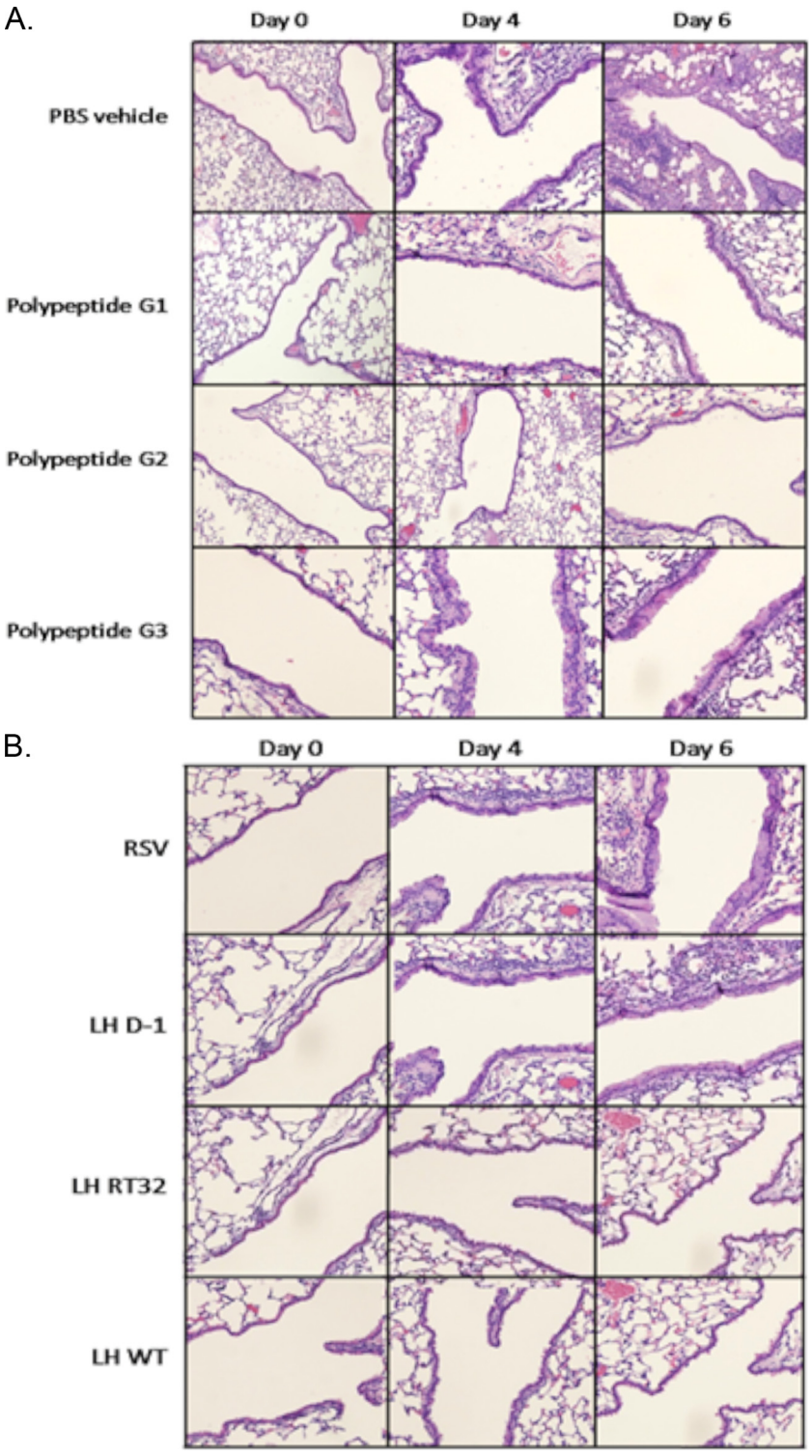

FIG. 7. Lung histopathology in mice vaccinated with G protein polypeptides or peptides and challenged with RSV. Lung histopathology was examined by hematoxylin and eosin staining in mice vaccinated i.m. with G protein polypeptides (G1, G2, and G3) or PBS vehicle (A) or vaccinated i.m. with G protein peptides (LH D-1, LH RT32, and LH WT) or RSV A2 (B). Mice were vaccinated once with G protein polypeptides or boosted twice, for G protein peptides, and subsequently were challenged i.n. with 10^6 PFU RSV A2 7 days after the last vaccination.

G protein peptide and polypeptide vaccination inhibits RSV replication. To determine if virus titers are associated with lung pathogenesis and body weight loss, the lung virus titers for the vaccinated groups of mice were determined at days 2, 4, and 6 post-RSV challenge (Fig. 8). The results from the studies were separated into two figures, i.e., Fig. 8A and B, to provide clarity, but they were performed together. Peak lung virus titers were detected at day 4 postinfection for all vaccination

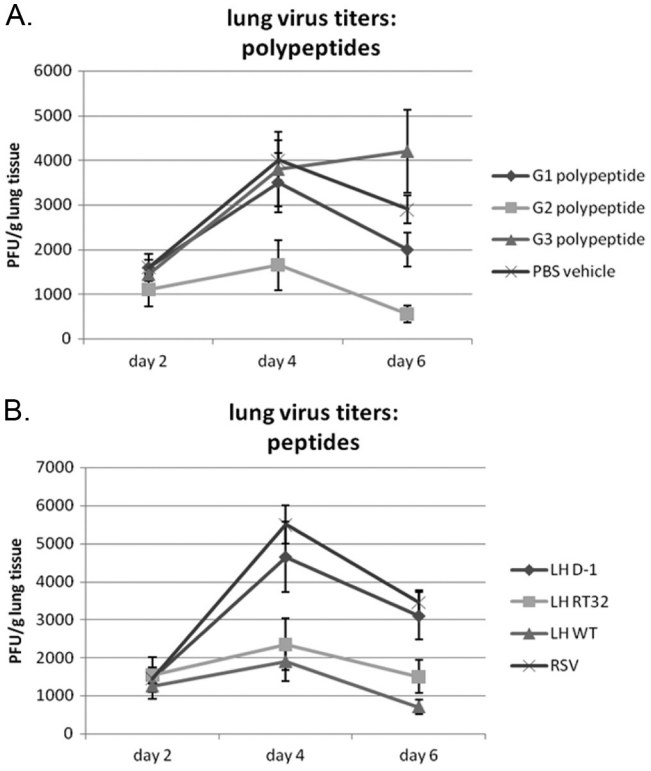

FIG. 8. Lung virus titers following RSV challenge of vaccinated mice. Mice vaccinated i.m. with G protein polypeptides (G1, G2, and G3) or PBS vehicle (A) or vaccinated i.m. with G protein peptides (LH D-1, RT32, and LH WT) or RSV (B) were challenged i.n. with 10^5 PFU RSV A2, and lung virus titers were determined at days 2, 4, and 6 postchallenge. Lung virus titers were determined by immunostaining plaque assay as previously described (57). Data are presented as PFU/g of lung tissue and are representative of two independent experiments.

groups, and titers generally declined by day 6 postinfection, but some mice vaccinated with G3 polypeptide showed evidence of continued virus replication at day 6 postchallenge (Fig. 8A). The G2 polypeptide-, LH WT peptide-, and LH RT32 peptide-vaccinated mice had significantly ($P < 0.05$) lower lung virus titers at days 4 and 6 post-RSV challenge than those of all other vaccination groups. These results suggest that vaccination with G2 polypeptide, LH WT peptide, or LH RT32 peptide vaccines induces protective antibodies that inhibit RSV replication and reduce disease pathogenesis associated with weight loss (Fig. 6) and lung histopathology (Fig. 7). Since antibodies also block G protein CX3C-CX3CR1 binding (Fig. 4), these results suggest that G protein CX3C-CX3CR1 blocking antibodies are protective in mice and prevent immune dysregulation through CX3C chemokine mimicry in RSV disease pathogenesis.

DISCUSSION

The development of a safe and effective RSV vaccine has been hindered by the young age at which RSV disease occurs, the experience of enhanced disease after vaccination using formalin-inactivated RSV, and the likelihood that virus-induced host immune responses contribute to disease pathogenesis. RSV vaccination at later times in life is also problematic because people experience reinfections throughout life (16, 22, 41), suggesting that natural infection does not lead to durable protection. These features of RSV disease and the failed efforts to develop a vaccine underline our lack of understanding of the constituents of a safe, protective immune response.

The RSV G and F proteins are the two major viral surface proteins associated with inducing the majority of protective immunity. The F protein has been shown to be most effective at inducing neutralizing antibodies and protective immunity in animal models (13, 14, 38, 52, 53, 58). The G protein also induces neutralizing antibodies and protective immunity (4, 25, 28, 36, 47, 49, 51), but it has features that contribute to RSV disease pathogenesis (5, 12, 29, 41, 54). One region in the RSV G protein contains a CX3C chemokine motif capable of binding to CX3CR1 and antagonizing the activities of CX3CL1 (56). This conserved CX3C region appears to be important for many disease-associated immune responses, as it has been shown to modify the immune response to RSV infection, facilitate RSV infection and persistence, and contribute to disease pathogenesis (23–26, 34, 55, 56). Interestingly, a variation in the CX3C receptor gene has been associated with increased risk for severe RSV bronchiolitis in children hospitalized for bronchiolitis (2), supporting the importance of G protein CX3C-CX3CR1 interaction in disease pathogenesis.

In this study, we demonstrate that the CX3C region of the G protein that is implicated in disease pathogenesis may also contribute to induction of a protective immune response by inducing antibodies that block G protein CX3C-CX3CR1 interaction. Our results suggest that antibodies that inhibit G protein binding to CX3CR1 also reduce parameters of disease pathogenesis, including weight loss and pulmonary inflammation, and inhibit lung virus replication more effectively than do antibodies generated to other G protein peptide vaccinations. Moreover, the blocking and protecting antibody responses to LH WT peptide vaccination are more effective than those of antibodies generated by vaccination with RT32 or LH D-1 peptide, suggesting that sequences in the central conserved region may be more effective than others in generating the desired antibody response. These findings are consistent with findings from our recent study showing that therapeutic treatment with a nonneutralizing monoclonal antibody specific to RSV G protein mediates virus clearance and decreases leukocyte trafficking and gamma interferon (IFN-γ) production in the lungs of RSV-infected mice (25, 35). The monoclonal antibody used in those studies does not bind at CX3C, but within the central conserved region of the G2 polypeptide region. In those studies, it was shown that the decrease in the pulmonary inflammatory response occurred independent of the presence of the Fc region on the antibody (35). Thus, we cannot assume that antibody-mediated neutralization has a dominant role in the current study.

A previous study from our group showed that anti-RSV G protein antibody responses in humans after recent RSV infection or vaccination are associated with inhibition of RSV G protein CX3C-CX3CR1 interaction and RSV G protein-mediated leukocyte chemotaxis, and the presence of these antibodies appeared to be associated with protection from RSV disease (24). The results from the present study similarly demonstrate that induction of antibodies by G protein peptides or polypeptides that contain the CX3C motif or by certain amino

acid sequences proximal to the CX3C region can inhibit G protein CX3C-CX3CR1 binding and RSV G protein-mediated leukocyte chemotaxis and prevent RSV disease pathogenesis. Taken together, these findings suggest that it may be possible to design a G protein polypeptide vaccine that, alone or combined with other RSV proteins, can induce a protective immune response without the disease-enhancing response otherwise associated with the G protein.

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